First isolation of *"Brachyspira hampsonii"* from pigs in Europe

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Abstract. Swine dysentery in Europe is classically attributed to *Brachyspira hyodysenteriae.* However, other *Brachyspira* species have been increasingly associated with intestinal disorders in pigs. This case report describes the first diagnosis of a *"Brachyspira hampsonii"* infection in European pigs. In a routine quarantine monitoring protocol, two gilts were presented for necropsy, in which soft watery non-haemorrhagic colonic content was found. Microbial culture from the colonic content and from faecal samples revealed the presence of strongly haemolytic, ring-phenomenon positive spirochetes indicative for *Brachyspira hyodysenteriae*. A diagnostic commercial PCR could not confirm the presence of *B. hyodysenteriae*. Phenotypic characterisation and PCRs targeting the 16S rRNA, 23S rRNA, *nox*, *hlyA* and *tlyA* genes of different swine-related *Brachyspira* spp. were performed. Phylogenetic analysis of sequences of the partial *nox* and 16S rRNA genes and multi locus sequence typing demonstrated that the isolates in this case were *"B. hampsonii"* isolates. This case report shows that the diagnosis of infections caused by new, emerging *Brachyspira* species is not self-evident and that the combination of microbial culture and PCR is recommended, completed with more extensive genotyping if necessary.

Key words:*Brachyspira;*swine dysentery;haemolysis;*"Brachyspira hampsonii"*

Case report: first isolation of *"Brachyspira hampsonii"* from pigs in Europe

Infections with *Brachyspira* spp. in swine occur in most swine-rearing countries and can result in substantial economic losses. Of all swine-related *Brachyspira* spp. infections classical swine dysentery, caused by *Brachyspira hyodysenteriae,* results in the most severe clinical symptoms (eg. mucohaemorrhagic diarrhea, weight loss, poor feed conversion). *B. hyodysenteriae* was first recognized as the cause of swine dysentery in 1971 (Taylor and Alexander 1971). At that time, the strong haemolysis of *B. hyodysenteriae* appeared indicative for pathogenicity since other, weakly haemolytic *Brachyspira* (formerly *Serpulina, Serpula* and *Treponema*) appeared to be commensal and were therefore named *Brachyspira innocens* (Kinyon and Harris 1979). Several reports of clinical disease caused by weakly hemolytic *Brachyspira* indicated that not all weakly hemolytic *Brachyspira* spp. were non-pathogenic for pigs (Taylor and others 1980, Neef and others 1994). Further research of these weakly haemolytic isolates including DNA-DNA hybridisation, resulted in the designation of three more weakly haemolytic species namely *B. intermedia, B. murdochii* and *B. pilosicoli* (Trott and others 1996, Stanton and others 1997).

These weakly haemolytic species of *Brachyspira* diverge in the severity of clinical symptoms they cause. *B. pilosicoli* is pathogenic and causes spirochetal colitis in pigs, which is marked by non-haemorrhagic diarrhea and a poor feed conversion. For *B. intermedia* and *B.murdochii* the pathogenic potential is less clear-cut. Although both species have been isolated from clinical cases of diarrhea, the clinical symptoms are mild or absent in experimental infections and yet high numbers of spirochetes are necessary to cause an effect (Jensen and others 2004, Jensen and others 2010).

Recently, a new type of *Brachyspira* infection has been described. Outbreaks of mucohaemorrhagic diarrhea, caused by strongly haemolytic *Brachyspira* strains inconsistent

with *B. hyodysenteriae,* were reported in the USA and Canada. Phylogenetic analysis of these strains showed such a large genetic divergence between those isolates and all other *Brachyspira* spp. that these isolates likely represent a novel species, for which the name *"Brachyspira hampsonii"* has been proposed (Chander and others 2012). The current case report describes, to the best of our knowledge, the first confirmed *"B. hampsonii"* infection in pigs outside North-America.

Two gilts, imported from the Czech Republic, were presented for necropsy in a routine quarantine monitoring protocol. General macroscopic findings consisted of a low body weight and dilated large intestines in which soft watery non-haemorrhagic colonic content was present. Histological examination of these large intestines was not performed. Microbial culture of the colonic content was performed on Tryptic Soy Agar (BD, Heidelberg, Germany) supplemented with 5% sheep blood (IMP, Brussels, Belgium), 0.1% yeast extract (Oxoid, Aalst, Belgium) and following antimicrobials: spectinomycin (200µg/ml), spiramycin (25 µg/ml), rifampin (12.5 µg/ml), colistin (6.25 µg/ml), and vancomycin (6.25µg/ml) (Hommez and others 1998). The microbial cultures revealed strongly haemolytic, ring phenomenon-positive spirochetes, indicative for *B. hyodysenteriae* (Fellström and others 1995, Hommez and others 1998). Some of the pigs, housed in the same group as the two gilts presented for necropsy, showed mild diarrhea. From the next batch of gilts from the same origin, additional faecal samples were taken in the quarantine. Strongly haemolytic *Brachyspira* isolates, with ring phenomenon, were again found on microbial culture, whereas commercial diagnostic PCR analysis (Adiavet Brachy, Paris, France) did not confirm the presence of *B. hyodysenteriae* in these samples. All faecal samples were negative for *Salmonella.*

Phenotypic characterisation tests were performed on pure cultures which were obtained by at least three subcultures on Tryptic Soy Agar (TSA) plates supplemented with

5% defibrinated sheep blood and 0,1% yeast extract (Jenkinson and Wingar, 1981). Phenotypic characterisation was performed on 4-day old cultures and was based on beta haemolysis, indole production, hippurate hydrolysis and the presence or absence of α-galactosidase, α-glucosidase and β-glucosidase (Fellström and others 1995). Indole production was determined using a spot-indole test (Remel BactiDrop, Dartford, UK) and for the other biochemical characteristics commercial discs were used according to the manufacturer's instructions (Rosco Diatabs, Taastrup, Denmark). Type strains of *B. hyodysenteriae* (ATCC 27164), *B. pilosicoli* (ATCC 51139) and *B.innocens* (ATCC 29796) were included to provide positive controls for all the phenotypic characteristics that were examined.

Several species-specific PCRs were performed, based on the following genes: *tlyA* (Råsbäck and others 2006), 23S rRNA (Leser and others 1997) and *nox* (Phillips and others 2006) for *B. hyodysenteriae, nox* (Phillips and others 2010) and 23s rRNA (Leser and others 1997) for *B. intermedia* , 16S rRNA (Phillips and others 2006) for *B. pilosicoli* and *nox* (Atyeo and others 1999) for *B.murdochii/B.innocens.* Additionally, PCR's were performed for the haemolysis related genes *hlyA* and *hlyA*-ACP (Barth and others 2012).

Forward primer 5' TAGCYTGCGGTATYGCWCTTT 3' and reverse primer 5' GCMTGWATAGCTTCRGCATGRT 3' were used to partially sequence the *nox* gene (Weissenböck and others 2005). A product of 1014 base pairs was obtained. Forward primer 5' GTTTGATYCTGGCTCAGARCKAACG 3' and reverse primer 5' CTTCCGGTACGGMTGCCTTGTTACG 3' were used to partially sequence the 16S rRNA gene of which a 1044 base pair product was obtained (Johansson and others 2004). Sequencing reactions were performed on purified PCR-product with the same primers as for PCR. *Nox* and 16S rRNA sequences from other *Brachyspira* isolates were retrieved from

GenBank and compared with the sequences of the described field case isolate (D52) by BLAST analysis.

The sequences of the *nox* gene of the strain retreived in this case report (D52), of *B. hyodysenteriae, B. intermedia, B. murdochii* and *B. innocens* ATCC type strains and of 42 additional strains of several *Brachyspira* spp. retrieved from GenBank were aligned using ClustalW. Sequences of clade I strain 30599 and clade II strain 30446 of *B. "hampsonii"* were also included (Rubin and others, 2013b). Phylogenetic analysis was performed with an alignment sequence fragment of 540 bp and Kimura distance calculation and neighbour-joining method were used.

For multilocus sequence typing (MLST) primers and PCR conditions as described by Råsbäck and others in 2007(b) were used to analyse genes encoding alcohol dehydrogenase (*adh),* esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and acetyl-coA acetyltransferase (*thi*). For each locus the sequence obtained from the D52 isolate was matched with the online MLSTdatabase (www.pubmlst.org/brachyspira).

The phenotypic characteristics of isolate D52 corresponded to those of *B. "hampsonii*" as described by Chander and others (2012). The isolate was strongly beta haemolytic, indole negative, hippurate negative, negative for α-galactosidase and α-glucosidase, and positive for β-glucosidase. Although not exclusively, most isolates of clade I are positive for β-glucosidase as compared to clade II, in which most isolates are negative for β-glucosidase.

Table 1 shows the PCR results. Isolate D52 generated a positive result in the two species-specific PCRs for *B. intermedia* based on the 23S rRNA and *nox* gene respectively. Interestingly, the PCR targeting *tlyA*, presumed typical for *B. hyodysenteriae*, also generated a positive result. The PCRs for several haemolysis associated genes, *hlyA* and ACP(fabF-fabG), were positive as well.

142 The *nox* sequence of isolate D52 (GenBank accession nr KF202498) showed a
143 similarity of 100% over 547 basepairs with *B. "hampsonii"* isolate NSH-16, which is
144 described by Chander and others as the reference strain of *B. "hampsonii"* clade I (Chander
145 and others 2012). Besides, the *nox* sequence of isolate D52 showed a similarity of more than
146 99% over 874 basepairs with previously described isolates KC35 en EB106 (JX197410.1 and
147 JX197409.1) (Burrough and others 2012b). These isolates, originally described as strongly
148 haemolytic *B. intermedia* are recently refered to as "*B. hampsonii* clade I" (GenBank). With
149 the type strain *B. hampsonii* 30599 (clade I, NZ_AOMM01000255.1) as described by Rubin
150 and others (2013b), the *nox* sequence of our isolate showed a similarity of 99% over 1014 bp
151 (difference of 2 nucleotides). The 16S rRNA sequence of isolate D52 (GenBank accession nr
152 KF586484) showed a sequence similarity of 99% over 1044 bp with "*B. hampsonii"* isolate
153 NSH-16.

154 Phylogenetic analysis of the *nox* sequence of isolate D52 and *nox* sequences of other
155 *Brachyspira* spp. clearly place isolate D52 in the cluster of isolates comprising clade I of "*B.*
156 *hampsonii"* (fig. 1)

157 As described for "*B. hampsonii"* in previous studies (Chander and others 2012) three
158 of the seven loci for MLST could not be amplified. From the sequences of the 4 loci that
159 could be amplified (*est, pgm, glp* and *thi*), none of them gave an exact match with known
160 alleles in the MLST database. The *thi* sequence matched closest with allele 24 of "*Serpulina*
161 sp. P280/1" (difference of 21 nucleotides) in accordance with the findings of Chander and
162 others, 2012, for "*B. hampsonii"*.

163 The results of the phenotypic characteristics, sequence comparisons, MLST and
164 phylogenetic analysis based on the *nox* sequence, identify the D52 isolate as "*B. hampsonii"*
165 clade I. To the best of our knowledge it is the first time that "*B. hampsonii"*isolates from

166 porcine origin are described in Europe, although the isolate *Serpulina* sp. P280/1 (Neef and
167 others 1994) in retrospect also may belong to "*B. hampsonii".*

168 The isolates of strain D52 obtained from the current field case, all contained the *hlyA*,
169 *tlyA* and ACP(*fabF*,*fabG*) genes. HlyA is the protein responsible for the strong haemolysis in
170 *B. hyodysenteriae* (Hsu and others 2001). In order to adequately perform its actions, the *hlyA*
171 gene has to be correctly placed between the accompanying *fab*-F and *fab*-G genes, coding for
172 an ACP-reductase and –synthetase (Zuerner and others 2004). Although the presence of *hlyA*
173 has been reported in some weakly haemolytic *Brachysira* spp. isolates, the *fabF* and *fabG*
174 genes were in those cases absent (Barth and others 2012), probably rendering the *hlyA* gene
175 functionally inactive. Another hemolysin, namely *tlyA* is consistently found in *B.*
176 *hyodysenteriae*. Although it has also been twice reported in weakly haemolytic species (Pati
177 and others 2010, Wanchanthuek and others 2010), these sequences show low sequence
178 similarity (82-83%) with *tlyA* of *B. hyodysenteriae* (Barth and others 2012). The presence of
179 both these hemolysin encoding genes in the isolates in the current field case may be
180 responsible for the strong haemolysis displayed by these isolates.

181 Rubin and others (2013a) could experimentally induce mucohaemorrhagic diarrhea in
182 swine when infected with a *"B. hampsonii"* strain 30446. The clinical signs were
183 indistinguishable from swine dysentery. It should, however, be noted that the strain 30446
184 clearly falls into the cluster II isolates of *"B. hampsonii"* whereas the strain from this case
185 falls into cluster I as shown in the phylogenetic tree in figure 1. Although experimentally "*B.*
186 *hampsonii"* strain 30599, which belongs to clade I, can induce severe clinical symptoms
187 (Harding and others, 2013), the symptoms in this case report were rather mild. This could be
188 due to difference in pathogenic potential between strains of clade I or be related to
189 *Brachyspira* colitis being a multifactorial disease. Environmental or nutritional factors may
190 alter the severity of clinical signs.

This case report and the recent case reports from Canada and USA (Burrough and others 2012a, Rubin and others 2013a) indicate that new, emerging species of *Brachyspira* can be important in swine-rearing countries. The results of the species-specific PCRs show that diagnosis of infections caused by these emerging species can be confusing. When diagnosis is solely based on microbial culture, all strongly haemolytic isolates will be reported as *B. hyodysenteriae*, whereas they could belong to the provisionally named species *"B. hampsonii"*, *"B. suanatina*" (Chander and others 2012, Burrough and others 2012a, Råsbäck and others 2007a, Rubin and others 2013a) or even other emerging *Brachyspira* species. On the other hand, when diagnosis is entirely based on PCR, strongly haemolytic isolates inconsistent with *B. hyodysenteriae* could easily be missed. For now, the combination of microbial culture and PCR, complemented with sequencing if necessary, is presumably the most complete method for diagnosis of *Brachyspira* spp. infections.

Declaration of conflicting interests

Sources of financial support have been acknowledged and the authors declare that they have no competing interests.

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330 **Tables and figures**

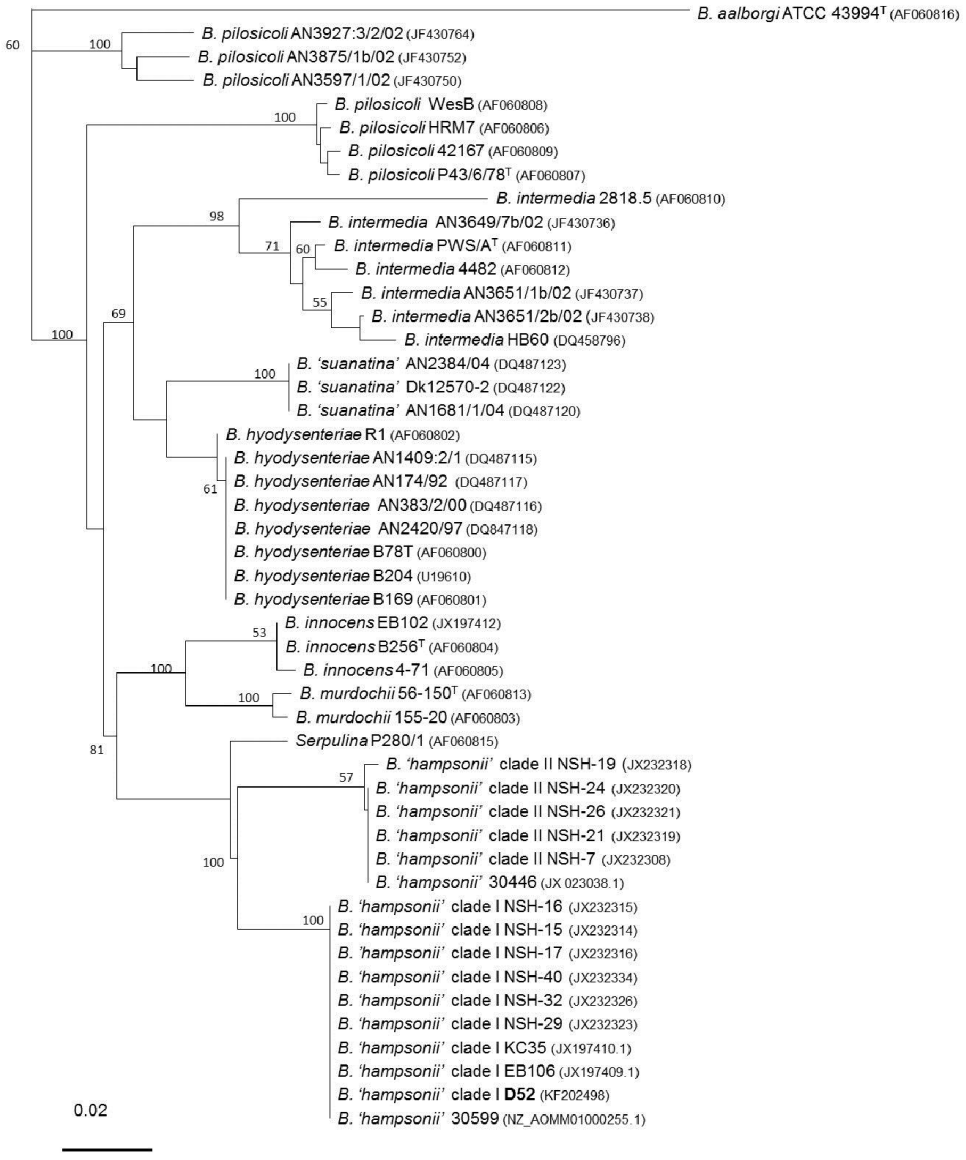
331 *Table 1:* Primers used in PCRs and results for *B.hyodysenteriae* reference strain ATCC 27164, *B. intermedia*

332 reference strain ATCC 51140 and the field case isolate D52.

Target Gene	**Species-specificity**	**Primer name**	**Primer Sequence (5'-3')**	**Result** D 52	**Result** ATCC 27164 *B.hyodysenteriae*	**Result** ATCC 51140 *B. intermedia*
hlyA	Non- specific	hlyAFo	TCG ATG AAA TTA AAG ATG TTG TT	positive	positive	positive
		hlyARe	TTT TTC TTG ATC TTC TTG AGG A			
ACP(fabF-fabG)	Non-specific	ACPFo	AGG IGA AGT IAT AGC IGT TGA CG	positive	positive	positive
		ACPRe	GAA ACA CCA TTA AGI AIA TTA TCC CA			
23S	*B. hyodysenteriae*	Hyo23SFo	CGG TAA GTG ATG TAC TTG	negative	positive	negative
		Hyo23SRe	AGC CTC AAC CTT AAA GA			
nox	*B. hyodysenteriae*	HyonoxFo	ACT AAA GAT CCT GAT GTA TTT G	negative	positive	negative
		HyonoxRe	CTA ATA AAC GTC TGC TGC			
tlyA	*B. hyodysenteriae*	tlyAFo	GCA GAT CTA AAG CAC AGG AT	positive	positive	negative
		tlyARe	GCC TTT TGA AAC ATC ACC TC			
nox	*B. intermedia*	IntnoxFo	AGA GTT TGA AGA CAC TTA TGA C	positive	negative	positive
		IntnoxRe	ATA AAC ATC AGG ATC TTT GC			
23S	*B. intermedia*	Int23SFo	CCG TTG AAG GTT TAC CGT G	positive	negative	positive
		Int23SRe	CGC CTG ACA ATG TCC GG			
16S	*B. pilosicoli*	Pilo16SFo	AGA GGA AAG TTT TTT CGC TTC	negative	negative	negative
		Pilo16SRe	GCA CCT ATG TTA AAC GTC CTT G			
nox	*B. innocens/ B. murdochii*	Innmurdnox Fo	CCT GAA AGT TTA AAA GCT G	negative	negative	negative
		Innmurdnox Re	CGA TGT ATT CTT CTT TTC C			

333

334 *Figure 1:* Phylogenetic tree based on the alignment (540bp) of the *nox* gene of *Brachyspira* spp. The
335 alignment was created using CLUSTALw, distance calculation (Kimura) and neighbour joining using PHYLIP.
336 Bootstrap values are indicated. Scale bar indicates 0,02 substitutions per site.


B. aalborgi ATCC 43994T (AF060816)
B. pilosicoli AN3927:3/2/02 (JF430764)
B. pilosicoli AN3875/1b/02 (JF430752)
B. pilosicoli AN3597/1/02 (JF430750)
B. pilosicoli WesB (AF060808)
B. pilosicoli HRM7 (AF060806)
B. pilosicoli 42167 (AF060809)
B. pilosicoli P43/6/78T (AF060807)
B. intermedia 2818.5 (AF060810)
B. intermedia AN3649/7b/02 (JF430736)
B. intermedia PWS/AT (AF060811)
B. intermedia 4482 (AF060812)
B. intermedia AN3651/1b/02 (JF430737)
B. intermedia AN3651/2b/02 (JF430738)
B. intermedia HB60 (DQ458796)
B. 'suanatina' AN2384/04 (DQ487123)
B. 'suanatina' Dk12570-2 (DQ487122)
B. 'suanatina' AN1681/1/04 (DQ487120)
B. hyodysenteriae R1 (AF060802)
B. hyodysenteriae AN1409:2/1 (DQ487115)
B. hyodysenteriae AN174/92 (DQ487117)
B. hyodysenteriae AN383/2/00 (DQ487116)
B. hyodysenteriae AN2420/97 (DQ847118)
B. hyodysenteriae B78T (AF060800)
B. hyodysenteriae B204 (U19610)
B. hyodysenteriae B169 (AF060801)
B. innocens EB102 (JX197412)
B. innocens B256T (AF060804)
B. innocens 4-71 (AF060805)
B. murdochii 56-150T (AF060813)
B. murdochii 155-20 (AF060803)
Serpulina P280/1 (AF060815)
B. 'hampsonii' clade II NSH-19 (JX232318)
B. 'hampsonii' clade II NSH-24 (JX232320)
B. 'hampsonii' clade II NSH-26 (JX232321)
B. 'hampsonii' clade II NSH-21 (JX232319)
B. 'hampsonii' clade II NSH-7 (JX232308)
B. 'hampsonii' 30446 (JX 023038.1)
B. 'hampsonii' clade I NSH-16 (JX232315)
B. 'hampsonii' clade I NSH-15 (JX232314)
B. 'hampsonii' clade I NSH-17 (JX232316)
B. 'hampsonii' clade I NSH-40 (JX232334)
B. 'hampsonii' clade I NSH-32 (JX232326)
B. 'hampsonii' clade I NSH-29 (JX232323)
B. 'hampsonii' clade I KC35 (JX197410.1)
B. 'hampsonii' clade I EB106 (JX197409.1)
B. 'hampsonii' clade I D52 (KF202498)
B. 'hampsonii' 30599 (NZ_AOMM01000255.1)
0.02

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